RESEARCH ARTICLE

No change in apoptosis in skeletal muscle exposed acutely or chronically to alcohol

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Abstract
The pathogenic mechanisms responsible for the deleterious changes in ethanol-exposed skeletal muscle are unknown, although apoptosis may be a causal process. We therefore investigated the responses of skeletal muscle to acute or chronic ethanol exposure in male Wistar rats. In acute studies, rats were dosed with ethanol (75 mmol (3.46 g)/kg BW) and killed after either 2.5 or 6 hours. In chronic studies, rats were fed ethanol as 35% of total dietary energy for 6 weeks. Apoptosis was determined by either DNA fragmentation or TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end labelling) assays. The results showed that apoptosis was not increased in the ethanol-exposed muscle in both acute and chronic studies compared to appropriate controls.

Introduction
Alcohol, one of the most commonly abused nutritional toxins, imposes profound metabolic and functional perturbations in skeletal muscle.1–3 These include poor contractile performance and muscular weakness, reductions in fibre diameter, loss of muscle bulk and impaired gait in affected subjects.4–7 Disruptions in a variety of biochemical processes also arise, such as impaired protein synthesis.8 The precise sequence of steps between alcohol exposure and the manifestation of alcohol-induced muscle diseases are, however, unknown. Nevertheless, several studies suggest that apoptosis may also play an important role in alcohol-induced pathologies.9–11 Apoptosis is an ATP-requiring process which cleaves cellular DNA, thereby physiologically adjusting and maintaining cell populations.12–15 Other morphological changes in apoptosis include shrinkage of cells,15 a hallmark of alcoholic myopathy.5–7 Thus, it is possible that apoptosis occurs in skeletal muscle exposed to ethanol. To test this hypothesis we measured apoptosis in muscle exposed to alcohol.
chronically for 6 weeks. We also examined acute ethanol exposure, i.e. 2.5 and 6 hours after ethanol administration. Short-term studies are important in elucidating pathogenic mechanisms in alcohol toxicity studies as they circumvent problems arising as a consequence of tolerance. Indeed, acute alcohol exposure may be an important initiating or precipitating step in alcohol-induced pathologies.5–7

Methods

Materials

Male Wistar rats were obtained from Charles River Ltd (Margate, Kent, UK). General and molecular biology grade chemicals were purchased from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) and Fisher Scientific (UK) Ltd (Loughborough, Leics, UK). RNAzol BTM was bought from Biotecx Laboratories Inc. (Houston, TX, USA). Agarose and molecular size markers came from Gibco-BRL Ltd (Life Sciences International, Paisley, Scotland, UK). The Puregene DNA isolation kit was from Gentra Systems Inc. (Minneapolis, USA). Ultrapure agarose, 10 × TBE buffer, the 123 bp ladder, HindIII digest and 1 kb ladder markers were from Life Technologies Ltd, Paisley, Scotland, UK. The Oncor non-organic DNA extraction kit and ApopTag in-situ apoptosis detection kits were from Oncor Inc., Gaithersburg, MD, USA. All other chemicals were obtained from Sigma-Aldrich Company Ltd, Poole, Dorset, UK.

Animals

Rats were housed in wire-bottomed cages in a constant temperature and humidity controlled animal house, with free access to commercial pelleted diet and tap water, until treatment. Animals were killed by decapitation at the relevant times. All studies were carried out in an Animal House approved by the Home Office and conducted under approved Personal and Project Licenses conforming to local and national ethical guidelines for the care and treatment of animals.

Acute ethanol experiment

Rats for the acute experiment were divided among the following groups, each of equal mean body weight (approx. 0.1 kg): (1) control (+ saline; 0.15 mol/l NaCl, injected i.p. 2.5 hours before sacrifice); (2) ethanol 2.5 hours (75 mmol (3.46 g)/kg body weight; injected i.p. 2.5 hours before sacrifice); (3) ethanol 6 hours (75 mmol (3.46 g)/kg body weight; injected i.p. 6 hours before sacrifice). Saline or ethanol was given as a single intraperitoneal injection as described previously by us.16 At 20 minutes and 1, 2.5, 6 and 24 hours, plasma levels of ethanol are approx. 450, 375, 290, 185 and 0 mg/100 ml, respectively.16 After treatment, the rats were sacrificed and the gastrocnemius rapidly dissected out and frozen in liquid nitrogen for subsequent analysis.

Chronic (6-week) ethanol feeding

When rats reached a body weight of approximately 0.1 kg they were divided into two groups (n = 8) of identical mean body weights and pair-matched according to body weight. They were then fed nutritionally complete liquid diets containing ethanol as 35% of dietary energy (i.e. treated rats) according to the Lieber–DeCarli protocol17 and described by us previously.18 The liquid diets were prepared daily from an enteral preparation (Fresubin). Control animals were pair-fed an identical amount of the same diet in which the ethanol had been replaced by an isoenergetic amount of glucose, as described previously for muscle studies.18–20 The feeding was carried out on an individual rat basis with the use of paired statistics for subsequent data analysis. At 6 weeks, rats were sacrificed and the gastrocnemius rapidly dissected out, then frozen in liquid nitrogen for subsequent analysis.

Using the above model of chronic alcohol exposure, there is a marked reduction in skeletal muscle weights as described previously by us.21–23 At 1, 2, 4 and 6 weeks, mean plasma levels of ethanol are 292, 364, 377 and 339 mg/100 ml, respectively.20 This is a standard regimen designed to produce a defined myopathic lesion as determined by reductions in fibre diameter and protein content with concomitant metabolic changes such as increased RNase activities, reduced protein synthesis and loss of ribosomal RNA.6,7,24,25

Measurement of DNA fragmentation by agarose gel electrophoresis

DNA extraction was performed using the Puregene DNA Isolation Kit, following the manufacturer’s instructions. Gel electrophoresis was carried out using a 1.8% (w/v) agarose gel.26
The gels were loaded with 10 μg DNA per lane and stained with ethidium bromide then photographed on an UV transilluminator. Quantification of the DNA fragmentation was performed using the UVP Gel Analysis Program. Following the instructions of the program, the 123-bp ladder was used to give a standard curve of the size of the bands against distance of migration. Bands corresponding to 180 bp and 360 bp in size are indicative of apoptosis. These were quantified in the gels. The extent of fragmentation was expressed as arbitrary densitometric units using data obtained for the areas under the curve; i.e. padu DNA; percentage of arbitrary densitometric units of total un-fragmented DNA. The reproducibility of the scanning was good (i.e. coefficient of variation of 9% for the same lane of agarose gel scanned six times), so we are confident in the scanning technique.

Detection of apoptotic cells by immunohistochemistry—the TUNEL method

The TUNEL [terminal dUTP (2′-deoxyuridine triphosphate) nick end labelling] method was used to detect apoptosis in gastrocnemius by immunohistochemistry, following the manufacturer’s instructions. At least 3000 myonuclei from three randomly chosen areas were counted for each tissue. The group identity of each tissue was not revealed until all counting had been performed.

Statistical analysis

After checking the normality of the data, statistical analysis for unpaired data was performed by Student’s t-test using a pooled estimate of variance for multiple comparisons. For paired data, Student’s paired t-test was used.

Results

Agarose gel electrophoretic analysis of DNA fragmentation

DNA fragmentation analysis showed no obvious laddering in muscle from rats given either saline or ethanol for 2.5, 6 hours or 6 weeks (Fig. 1). No obvious DNA laddering could be seen due to ethanol treatment (Fig. 1). This was also evident when gels were scanned in order to attenuate any 180 bp and 360 bp fragments that were not discernable visually (Table 1). We could not find any difference between the relative abundance of 180 and 360 bp fragments in either control or ethanol-exposed muscle (Table 1).

Detection of apoptotic cells by immunohistochemistry—the TUNEL method

Representative slide photographs of the TUNEL method are shown in Fig. 2. The positive control in Fig. 2 shows a rat mammary gland at the fourth day after weaning. The negative control was prepared by substituting the TdT enzyme for distilled water. Only a few apoptotic nuclei were found in the muscles (Table 2 and Figure 2), confirming the data in Table 1. Although at least three areas of tissue were used to count apoptotic nuclei, in many instances, entire sections of muscle slides were also examined only to ascertain that one or two nuclei were apoptotic, or none at all. We believe that the estimate of less than 0.05% of cells being apoptotic is accurate (Table 2).

Discussion

Skeletal muscle pathologies in alcoholism are important metabolically as this tissue comprises 40% of body mass and a quarter of whole body protein synthesis. Furthermore, alcohol ingestion can also be considered from a nutritional perspective due to its impact on nutrient handling, wasting, and its displacement of nutrient-rich calories, to name but a few examples. It was our original hypothesis that alcohol induces apoptosis in skeletal muscle. There are three lines of circumstantial evidence to support this. First, various cells and organs, such as thymocytes, liver and brain undergo apoptosis in response to ethanol. Secondly, there is some evidence to suggest that apoptosis is increased as a consequence of oxidative stress. We have demonstrated previously that alcohol administration to rats increases oxidative stress in skeletal muscle in both acute and chronic studies. Thus, by analogy it is possible that the occurrence of oxidative stress and/or increased generation of reactive oxygen species may well initiate apoptosis in skeletal muscle from alcohol-exposed tissues. Finally, it is noteworthy that a variety of myopathies characterized by functional or metabolic impairments in skeletal muscle are associated with increased apoptosis and it is
possible that similar processes occur in alcoholic myopathy.

Conceptually, understanding the contribution of apoptosis to alcoholic skeletal myopathy is important as it may provide the focus for novel therapeutic strategies in situations where abstinence has failed or when there is marked metabolic repercussions from alcohol-induced organ injury. Targeting the apoptotic pathways in such scenarios have previously been proposed for alcoholic liver disease. Furthermore, it also possible that some of the pathogenic mechanisms in alcoholic myopathy may also be applicable to other toxic or metabolic myopathies characterized by muscle weakness and wasting. However, we were unable to detect any increased apoptosis in

![Figure 1. Effect of acute and chronic (6 week) ethanol administration on DNA laddering in the rat gastrocnemius. Lane 1 = 123 bp ladder; lane 2 = lambda/HindIII digest; lane 3 = negative control; CA = 2.5 hours saline; E2.5 = 2.5 hours ethanol; E6 = 6 hours ethanol; CC = control groups in chronic (6-week) ethanol study; E6 = chronic (6-week) ethanol-fed groups. DNA laddering was compared by agarose gel electrophoresis. There was no overt laddering in any of the groups.](image)
In the subsequent discussion we focus on the methodological aspects of our experiments.

**Methodological considerations**

We examined both acute and chronic alcohol exposure in order to circumvent the possibility that the potential failure to see any apoptosis was due to the development of tolerance in chronic studies or because the periods of exposure were limiting in acute experiments. These experimental times (2.5 hours, 6 hours and 6 weeks) were based on our long-standing studies in skeletal muscle where we have been able to show either loss of contractile elements or proteins, impaired protein synthesis, increased oxidative stress or other pathological indices in muscle at these time-points.\(^5,6\) It is important to point out that the gastrocnemius displays similar biophysical features to the plantaris, including its Type II fibre predominance.\(^45,46\) In terms of its response to alcohol, the gastrocnemius is very similar to the behaviour of the entire muscle mass.\(^21\) These factors, together with its large size, make this muscle amenable for biochemical analysis when the amount of material in such smaller muscles such as the plantaris is limiting.\(^47\)

We employed two methods for detecting any increased apoptosis, namely DNA fragmentation or laddering and the TUNEL assay. DNA fragmentation or laddering is based on the cleavage of DNA between nucleosomal units.\(^15\) This generates mono- and oligo-nucleosomal fragments in multiples of 180 bp units.\(^15\) It could be argued that the time-points of the experimental studies were inappropriate. However, DNA fragmentation increases within 2 hours of glucocorticoid treatment of rat thymus.\(^48\) In avian thymocytes, glucocorticoid treatment for 4 hours increases DNA fragmentation.\(^49\) Thus, we conclude that the time-frame for our studies on the DNA laddering technique were appropriate for addressing the original hypothesis.

In our immunological studies over 3000 myonuclei from three randomly chosen areas were counted for each tissue. Previous studies have counted 450 Y-79 retinoblastoma cells after TUNEL\(^50\) or 300 cells for each sample of bovine aortic endothelial cells.\(^51\) The seven- to 10-fold higher number of myonuclei that were counted (3000) in these experiments was thus deemed sufficient to give an accurate estimation of the percentage of apoptotic cells, if this was increased in response to ethanol. The group identity of each tissue was not revealed until all counting had been performed, so that results were not biased. Only the areas of the tissue that contained

<table>
<thead>
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<th>Table 1. Effect of acute and chronic ethanol administration on DNA laddering in the rat gastrocnemius</th>
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<td>Acute treatments</td>
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<tr>
<td>Control, 2.5-hour saline</td>
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<tr>
<td>Ethanol, 2.5-hour</td>
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<tr>
<td>Ethanol, 6-hour</td>
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<tr>
<td>Control, 6-week glucose</td>
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<td>Treated, 6-week ethanol</td>
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The amount of 180 and 360 bp DNA fragments in control or ethanol-treated rats were expressed as a percentage of arbitrary densitometric units of total unfragmented DNA (i.e. % padu DNA). NFD, no fragments detected by densitometric analysis. Mean ± SEM values are displayed. There were no significant differences between any of the control and treated groups in either acute or chronic studies (p > 0.05 for either 180 and 360 bps in all instances).
myonuclei were investigated. Of course, it could be argued that apoptosis in skeletal muscle is not subjected to acute regulation and periods longer than 6 weeks may be necessary. However, this supposition is not supported by the observation that this period of feeding produces defined myopathological lesions nor by the fact that increases in TUNEL-positive cells are discernable in skeletal muscle after only 1–3 hours within the initiation of apoptosis.  

Of note was the observation that we recorded by scanning 180 and 360 bp fragments in muscle DNA of control rats fed liquid diets for 6 weeks (Table 2) whereas these fragments were relatively absent from scanned gels prepared from control muscles from the acute loading study (Table 1). We are unsure why this occurred but it may be

**Table 2. Effect of acute and chronic ethanol administration on the percentage of apoptotic cells as determined by the TUNEL assay**

<table>
<thead>
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<th>Treatment</th>
<th>Mean percentage of apoptotic cells (%)</th>
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<tbody>
<tr>
<td><strong>Acute studies</strong></td>
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<tr>
<td>Control, 2.5-hour saline (4)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Ethanol, 6-hour (4)</td>
<td>&lt; 0.05</td>
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<tr>
<td>Ethanol, 2.5-hour (6)</td>
<td>&lt; 0.05</td>
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<tr>
<td><strong>Chronic studies</strong></td>
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<tr>
<td>Control, 6-week glucose (8)</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Treated, 6-week ethanol (8)</td>
<td>&lt; 0.05</td>
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The mean number of apoptotic cells in the gastrocnemius muscle of acutely and chronically treated rats were determined in at least 3000 nuclei per rat sample.
due to differences in either the age of the rats, malnutrition or some other factor. For example, at the end of these chronic feeding studies rats were approximately 3 months old compared to those described in the acute studies which were approximately 1.5 months old. Ageing has been shown to increase apoptosis in skeletal muscle.\textsuperscript{15} Rats in the chronic study were also marginally malnourished due to the imposition of the pair feeding, as reviewed previously.\textsuperscript{20} Starvation has also been shown to increase apoptosis in muscle cells.\textsuperscript{52} However, it would be imprudent to make strict comparisons between the control rats in the acute and chronic studies as both were fed on different diets. For example, a solid chow diet was fed to the rats used in the acute study compared to the liquid diet which was fed to rats in the chronic study. Nevertheless, we are confident in our conclusions that there was no increased DNA laddering in response to alcohol exposure as both control and ethanol treated rats were analysed simultaneously using the same protocols for each study.

Alcohol has been associated with apoptosis in various cells types, but most frequently in hepatic\textsuperscript{36,53,54} and neuronal tissue.\textsuperscript{10,55} More recently, indirect pathways for ethanol in promoting apoptosis have been described such as the enhancement of oxysterol-induced cell death in the endothelium.\textsuperscript{56} Alcohol may also exert its effects on apoptosis via the intermediate acetaldehyde.\textsuperscript{57} However, there is no evidence in the literature of apoptosis due to ethanol in skeletal muscle. It is important to emphasize that other methods have also been adopted for determining apoptosis including immunolocalization of Bcl-2, Bax-Xl, aspects of the caspase – apoptotic signaling pathways or cytochrome c release.\textsuperscript{15} In alcohol toxicity Fas/Apo-1 receptor expression or annexin-V binding have also been measured (for example, see Cheema \textit{et al.} \textsuperscript{11}). However, these are indirect methods.\textsuperscript{15} Moreover, we were focused primarily in addressing the issue of whether apoptosis \textit{per se} was increased, rather than whether other aspects of the apoptotic signaling pathways were active. It is generally accepted that the final evidence of apoptosis is obtained from assays using TUNEL or DNA fragmentation whereas other methods (such as the aforementioned) are either indirect and give only an indication of the pathways leading to the final step of programmed cell death.\textsuperscript{15} None the less, neither DNA fragmentation nor TUNEL assays demonstrated apoptosis in response to ethanol and we conclude that other processes must contribute to the metabolic and functional lesions seen in this tissue.

We are confident that we used the most appropriate models of alcohol-induced muscle disease. For example, acute ethanol dosage reduces muscle protein synthesis whereas in the 6-week chronic model, there is a marked reduction in muscle weights.\textsuperscript{6,7,24,25} Although we have no data on the distribution of ethanol in muscle and liver, recent evidence suggests that alcohol \textit{per se} may not be the more important causative agent. For example, acetaldehyde seems to play a crucial role in reducing muscle protein synthesis.\textsuperscript{6,7,24,25} A further example of the concept that ethanol \textit{per se} should not be considered alone has been obtained from rats in which endocrine status has been manipulated: modification of hormonal status modulates the effects of ethanol.\textsuperscript{58}

A number of studies have shown that there is an important relationship between apoptosis and skeletal muscle disorders, such as cancer cachexia,\textsuperscript{59,60} denervation,\textsuperscript{61} steroid myopathy,\textsuperscript{62} Duchenne muscular dystrophy,\textsuperscript{63} and cardiac cachexia.\textsuperscript{64} However, we believe that mechanisms controlling atrophy in other myopathies may not be applicable to alcohol-induced muscle disease:65 The present results support this supposition.

\textbf{Acknowledgements}

This work was supported by the Wellcome Trust.

\textbf{References}


